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PRINCIPAL INVESTIGATOR: Francis G. Kern, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
37th and O Streets, NW
Washington, DC 20057

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Markers of Fibroblast Growth Factor Family-Mediated Growth Signal Transduction as Determinants of Successful Hormonal Therapy for Patients with Estrogen Receptor Positive Breast Cancer

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Francis G. Kern, Ph.D.

Georgetown University
37th and O Streets, NW
Washington DC 20057

U.S. Army Medical Research, Development,
Acquisition and Logistics Command (Provisional)
Fort Detrick, Frederick, Maryland 21702-5012

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13. ABSTRACT (Maximum 200 words)

Previous work from the laboratory sponsored by this award demonstrated that overexpression of two members of the fibroblast growth factor (FGF) family of cytokines in a estrogen-dependent human breast cancer cell line resulted in the cancer cells acquiring the ability to grow in mice that were treated with the antiestrogen tamoxifen. Other work demonstrated the presence of high levels mRNA for FGFR-3, one of the transmembrane tyrosine kinase growth factor receptors for FGFs, in a subset of human breast tumors. During the course of the period covered by this report, two high titer polyclonal rabbit antibodies were generated against two peptides present in the FGFR-3 protein. Pathological specimens from 181 patients with estrogen receptor positive breast tumors that received tamoxifen therapy were identified. The suitablity of the FGFR-3 antibodies for use in immunohistochemical assays with sections from formalin-fixed and paraffin-embedded material from these patients is currently being evaluated. Reverse transcription- polymerase chain reaction (RT-PCR) was used to generate plasmid transcription vectors for FGF-7 and a FGFR-2 splice variant that generates a specific receptor for FGF-7. These vectors were used to prepare probes for RNAse protection assays for FGF-7 and its receptor. Although the probe for the receptor did not appear to be suitable for the assay, the presence of FGF-7 RNA was detected in 35 of 36 human breast tumors examined.

14. SUBJECT TERMS

breast cancer, antiestrogen resistance, fibroblast growth factors, growth factor receptors

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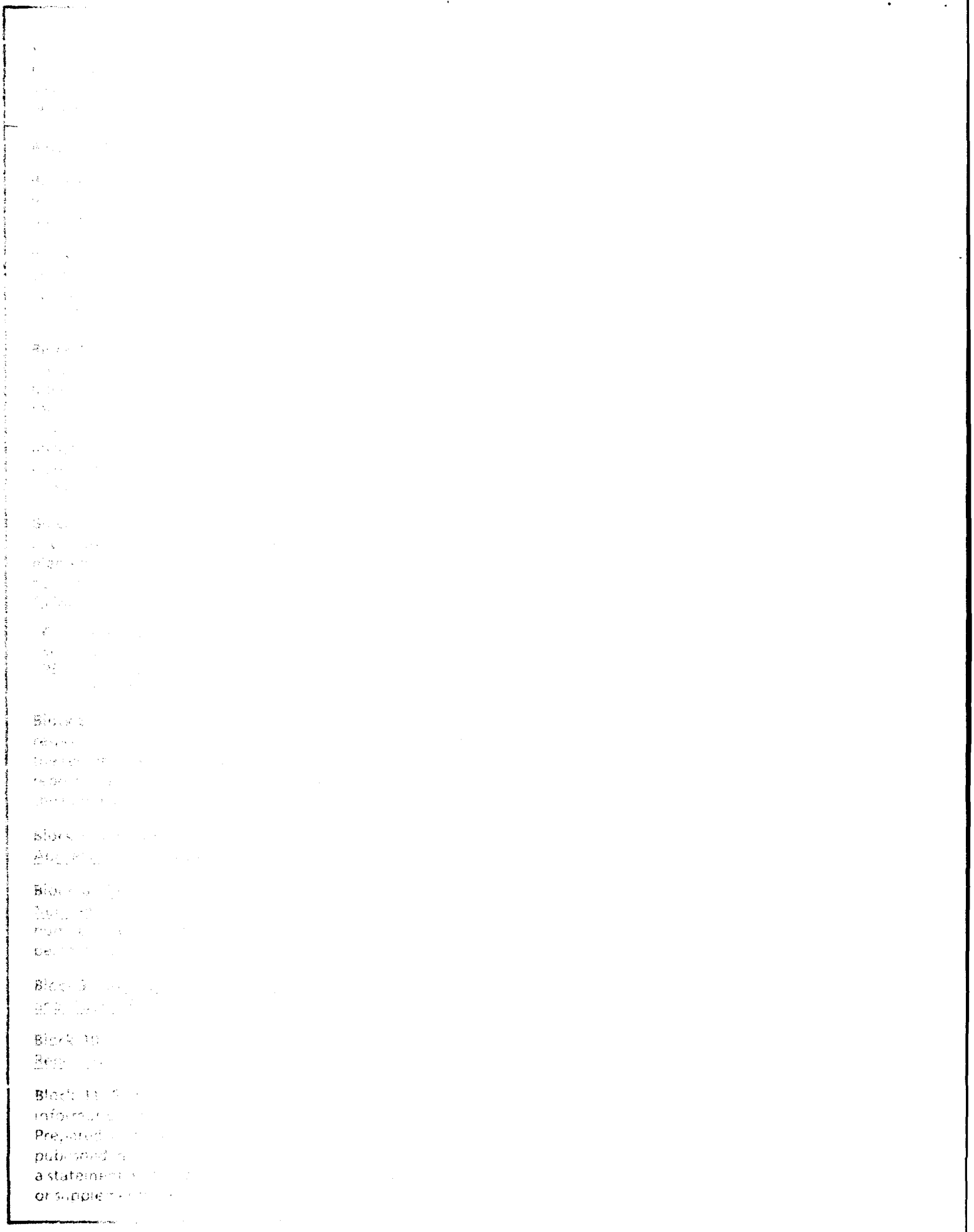

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INTRODUCTION:

A response to tamoxifen therapy is observed in less than 60% of breast cancer patients with recurrent disease that initially presented with estrogen receptor positive tumors. Therefore additional markers are needed that will allow the clinician to better predict that subpopulation of patients most likely to respond to this therapy. Our laboratory has demonstrated that overexpression of two members of the fibroblast growth factor family in an estrogen receptor positive breast cancer cell line confers these cells with the ability to grow tumors in ovariectomized athymic nude mice treated with tamoxifen (1). These results suggest that FGF signal transduction mediated through FGF binding to specific transmembrane tyrosine kinase receptors may be capable of overcoming the estrogen dependence of estrogen receptor positive (ER+) breast cancers for growth. Indicators of FGF signal transduction being operative within an estrogen receptor positive tumor might therefore be capable of providing a sign of the likelihood that tamoxifen therapy will be ineffective. These indicators could include evidence of expression of either FGF ligands or FGF receptors. Nine members of the FGF family of ligands have been identified to date and four transmembrane tyrosine kinase receptors activated by ligand binding have also been described (2-5). We have used sensitive and specific RNAse protection assays to examine the range of expression of six of the nine FGF ligands and all four receptors in human breast tumors. With such an assay we have found that FGFR-3 is highly expressed in a subset of tumors (6). One objective of this project included raising antibodies that could be used in an immunohistochemical assay for FGFR-3 expression. Such antibodies would then be used to perform a retrospective study on formalin fixed and paraffin embedded material from patients with ER+ tumors that received tamoxifen treatment and for whom follow up information could be retrieved. This would allow the one to determine if FGFR-3 overexpression correlates with the failure of tamoxifen therapy. A second objective was to develop RNA probes that could be used to examine the range of expression of FGF-7 expression and its receptor in human breast cancers.

BODY:

A. Preparation of polyclonal rabbit antesera against FGFR-3

1. Choice of peptide antigens. Two hydrophilic, extracellular portions of FGFR3 were chosen as potential antigens using the PlotStructure and Helicalwheel programs in the GCG software package, and using the Antigen program in the PCGENE software package. They are (1) peptide-a: RAAEVPGPEPGQQEQ, corresponding to amino acids 33-48 (15-mer) of FGFR-3 and (2) peptide-b: PQRLQVLNASMEDS, corresponding to amino acids 91-105 (14-mer) of FGFR-3. These two hydrophilic and extracellular portions where also chosen based on there limited homology to corresponding regions in FGFR-1, FGFR-2 and FGFR-4. 50 mg of HPLC purified peptide-a and peptide-b were synthesed by BioServ, Labs (San Jose, CA).

2. Peptide coupling and rabbit immunization. An additional cysteine residue was attached to the carboxy terminus of both peptides during synthesis to facilitate coupling to carrier. 25 mg of peptide-a and peptide-b were conjugated to Keyhole limpet hemocyanin (KLH) using the heterobifunctional reagent, m-Malemidobenzoyl-N-hydroxysuccinimide (MBS). For each KLH-coupled peptide, two New Zealand white rabbits, female, weight 3 kg, were immunized by subcutaneous injections into

multiple sites on the rabbit's back. The first immunization was performed with 0.5 mg of peptide-KLH and subsequent boosts were performed with 1mg of peptide-KLH. Complete Freund's adjuvant (GIBCO) was used in the first immunization and incomplete Freund's adjuvant was used in later boosts. The ratio of antigen: adjuvant was 1:1 (v/v). The animals received their first boost three weeks after the primary injection and subequent boosts were continuously carried out at intervals of 14 days. A prebleed was done immediately before the first immunization. Bleeds from the ear vein and artery were carried out 7 days after boosts.

3. Determination of the titer of anti-FGFR3 in immunized rabbit serum. An enzymel linked immuno absorbant assay (ELISA) for the titer of anti-FGFR-3 was developed as follows. 200 ng/well of peptide-a or peptide-b was added to each well of a 96 well of Reacti-Bind™ Maleic Anhydride Activated Polystyrene Plates (PIERCE) and incubated at 37°C overnight. After washing, 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) was added at room temperature for 1 hour followed by washing with PBS with 0.1% BSA and 0.05% Tween-20 9wash buffer). The rabbit serum was diluted at 1: 6,000; 1:12,000; 1:24,000; 1: 48,000 in 0.5% BSA-PBS and 200 ul of the diluted immunized rabbit serum was added to the well to allow the anti-FGFR-3 peptide antibodies to bind to corresponding peptide. After incubation at room temperature for 1 hour, the nonspecific protein in the serum was removed by three washes with wash buffer. 200 ul of 1:2000 diluted horseradish peroxidase (HRP) conjugated sheep anti-rabbit IgG was added to the wells and further incubated for 1 hour at room temperature. After three washes to remove unbound second antibodies, 200 ul of HRP substrate, 3,3',5,5' Tetramethyl Benzidine (TMB, 0.4g/L, PIERCE) was added to the well to develop the color. The reaction was stopped with 100 ul of 2M H_2SO_4 The plates was read at A_{450} in Auto-ELISA reader. The prebleed serum at the same dilution was used as background control. The results of the ELISA assays of anti-FGFR-3 from 4 rabbits is included in Figure 1 and Figure 2 in the Appendix. They indicate that for all four sera, positive titers were still obtained at the highest dilution tested (1:512,000).

4. Development of an immunohistochemical assay for FGFR-3.

Information contained within the Georgetown University Medical Center tumor registry was used to generate a list of 181 patients that received tamoxifen therapy. Pathological specimens from approximately 160 of these patients are available through the Georgetown University Medical Center Pathology Department. We are currently in the process of identifying the paraffin blocks from each of these patients that contains breast tumor tissue. Once identified, microscopic sections from each block will be prepared.

B. Development of RNAse protection assays for FGF-7 (a.k.a. keratinocyte growth factor or KGF) and the KGF receptor (KGFR) isoform

1. FGF-7. A 195 bp partial cDNA starting from the start codon covering the first 65 amino acids of FGF-7 was synthesized using reverse transcription-polymerase chain reaction (RT-PCR). This portion was chosen based on its relative lack of homology within this region to corresponding regions of FGFs 1-6. The PCR primers utilized were sense : 5'-ATCCACAAATGGATACTGAC-3' and antisense: 5'-TTATATCCCCTCCTTCCATG-3. The template for RT-PCR was human placental RNA. The reverse transcription and PCR reactions were performed according to the

instructions provided by the manufacturer of the RT-PCR kit (Perkin-Elmer). The PCR product was made blunt-ended and ligated into the pGEM7zf+ transcription vector that was linearized with SmaI. the sequence of plasmid inserts from ampicillin resistant colonies arising after transformation was verified by dideoxy sequencing using the T7 P/P primer (Promega). A plasmid having the insert in the orientation: 5'-T7---KGF sense (1-195 bp)---SP6-3' was chosen and plasmid DNA was linearized with EcoRI. A 283 nucleotide P-32 labeled antisense transcript was prepared and RNAse protection assays were performed as described in (1). RNA was isolated from a number of human breast cancer cell lines and 36 human breast tumor tissues using either guanidium isothiocyanate or RNAzol methods. RNA from the human WI-38 Embryonic lung fibroblast cell line obtained from the ATCC was used as a positive control. All human breast cancer cell lines were negative for expression of FGF-7 mRNA. However, 35 of 36 breast tumor tissues tested were positive in the assay. A photocopy of a representative experiment with breast tumor RNA is included as Figure 3 in the Appendix.

2. Generation of a riboprobe for the FGFR-2 exon IIIc splice variant. Exon shuffling within the third IgG-like domain of FGFR-2 generates a receptor isoform (KGFR) that is capable of binding FGF-7 (KGF) with much higher afffinity (5,7). RT-PCR was also used to generate a 171 bp cDNA corresponding to nucleotides 927 to 1098 of the FGFR-2 exon IIIc cDNA, the region that that contains this exon. The primers used were: sense : 5'-CAAGGTTCTCAAGCACTCGGGGATAAATAGTTCCAA-3', and antisense: 5'-CTTCCAGGCGCTTGCTGTTTTGGCAGG-3'. RNA from the MCF-7 ER+ breast carcinoma cell line was used as the template for the reverse transcription. The conditions for PCR were 94°C, 1 min 60°C, 2 min; 72°C, 1 min; 35 cycles. The PCR product was blunt-ended and ligated to SmaI digested pGEM7zf+ DNA and insert sequences were confirmed by dideoxysequencing. A plasmid with the orientation: 5'-T7---KGFR antisense ---SP6-3' was chosen for use. After linearization with Hind III, a 251 nucleotide riboprobe was generated using T7 polymerase. However conditions for RNAse protection could not be successfully established with this probe and an artefactual band was consistently observed.

CONCLUSIONS:

High titer FGFR-3 rabbit antisera have successfully been generated and a suitable number of patient samples have been identified. For immunohistochemical assay development we will need positive and negative controls. For this purpose we will use human breast tumors grown in athymic nude mice. The MDA-MB-231 cell line expresses very low levels of RNA for FGFR-3 (8) and therefore the amount of protein produced should be below the detection limits of the assay. Sections from formalin fixed and paraffin embedded MDA MB231 tumors can therefore be used as negative controls. We are currently in the process of constructing a eukaryotic expression vector containing the full length FGFR-3 cDNA. This vector will be used to generate tumorigenic cell lines that overexpress FGFR-3 for use as a positive control.

A successful RNAse protection assay for FGF-7 mRNA expression has been developed. The detection of FGF-7 mRNA in human breast tumor tissues but not in any human breast cancer cell lines implies that the expression emanates from he stromal component of the tumor. The probe for KGFR expression was not found to be useful. An RT-PCR assay for KGFR expression in human

tumors is currently being developed as an alternative.

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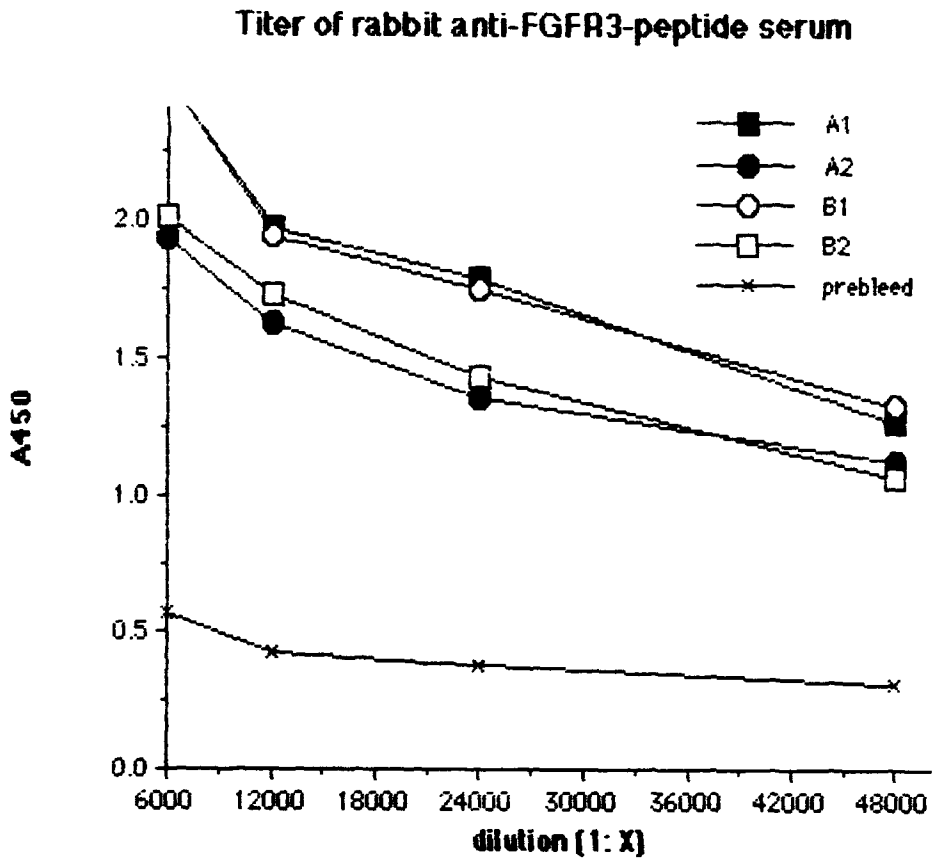
Titer of rabbit anti-FGFR3-peptide serum
A1
A2
B1
B2
prebleed
A450
2.0
1.5
1.0
0.5
0.0
6000
12000
18000
24000
30000
36000
42000
48000
dilution (1: X)

Figure 1

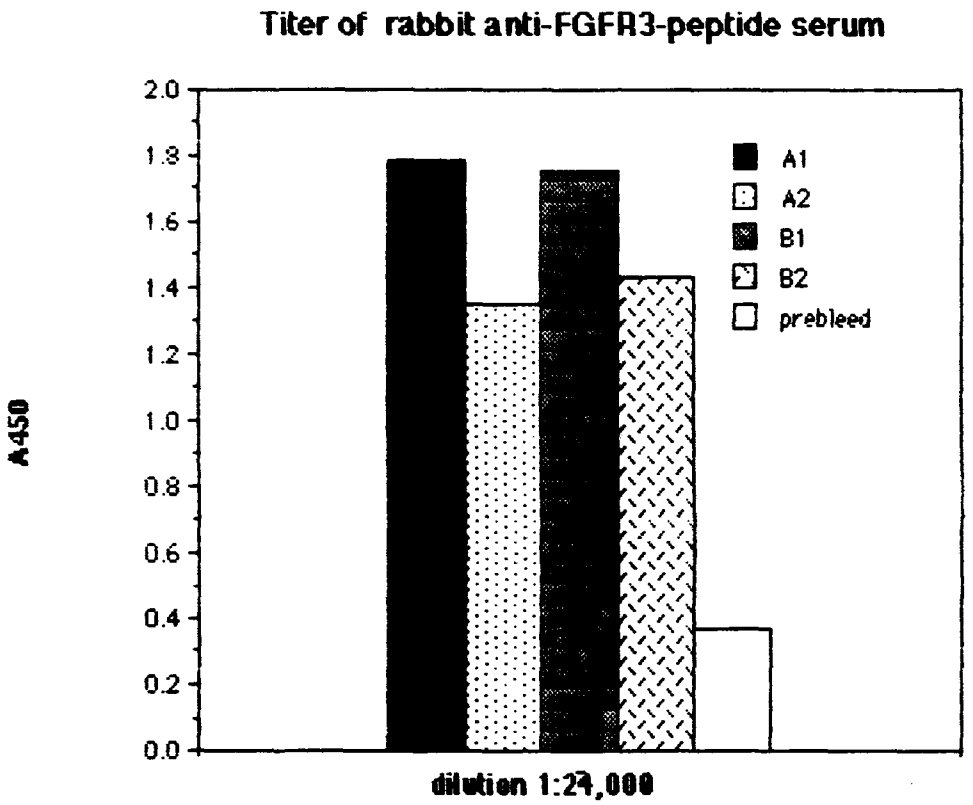
Titer of rabbit anti-FGFR3-peptide serum
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A450
A1
A2
B1
B2
prebleed
dilution 1:24,000

Figure 2

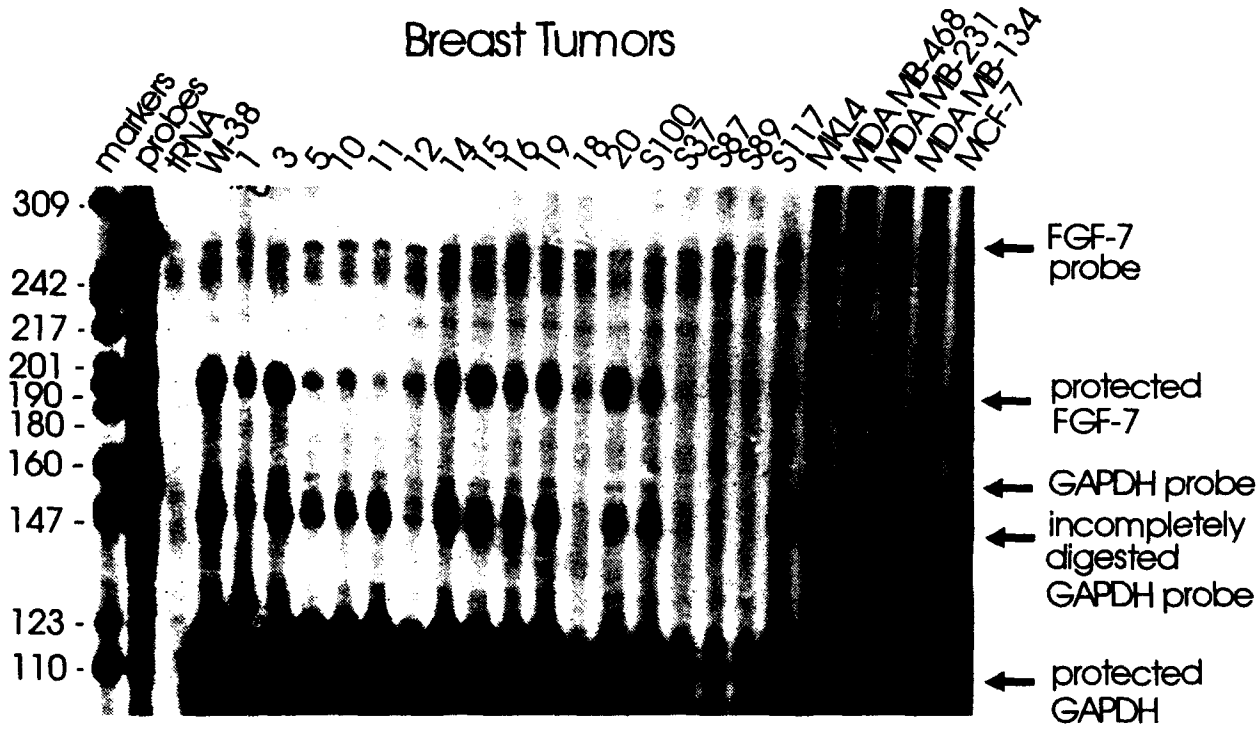

Figure 3. RNAse protection assay demonstrating expression of FGF-7 (KGF) mRNA in Human Breast Tumors. A riboprobe for glyceraldehyde phosphate dehydrogenase (GAPDH) was included in the hybridization cocktail to control for differences in loading.